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PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 1/21, 15/21, 15/09
C12N 15/76, 15/67

A1

(11) International Publication Number: WO 91/06628

(43) International Publication Date: 16 May 1991 (16.05.91)

(21) International Application Number: PCT/US90/06081

(22) International Filing Date: 26 October 1990 (26.10.90)

(30) Priority data:
429,092 30 October 1989 (30.10.89) US

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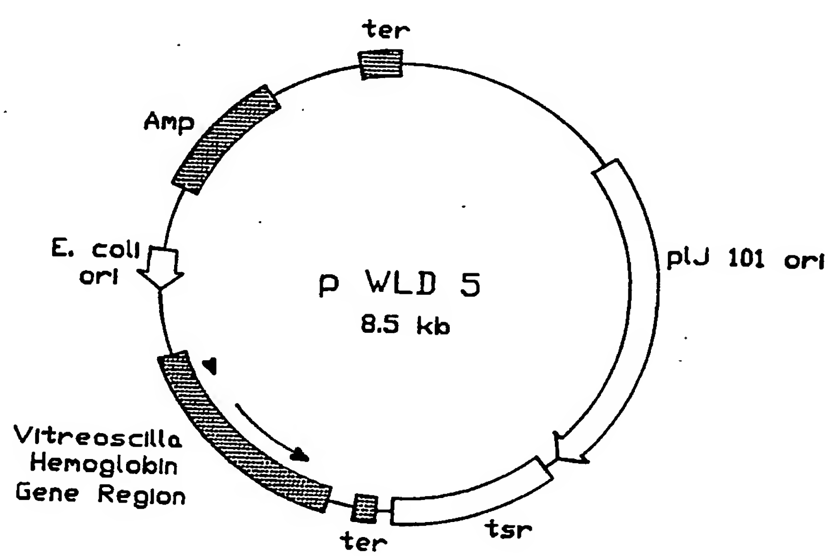
(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: EXPRESSION OF BACTERIAL HEMOGLOBIN AND ENHANCEMENT OF EXPRESSION OF CLONED AND NATIVE PRODUCTS IN STREPTOMYCES



(57) Abstract

The invention relates to a method for expressing bacterial hemoglobin in *Streptomyces*, and, by this means, for enhancing aerobic metabolism and antibiotic production in *Streptomyces*. The invention also relates to expression vectors for expression of native and heterologous genes in *Streptomyces*. Exemplary vectors pWLD5 and pWLD10 are shown in the figures.

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EXPRESSION OF BACTERIAL HEMOGLOBIN AND ENHANCEMENT OF EXPRESSION OF CLONED AND NATIVE PRODUCTS IN STREPTOMYCES

This is related to Serial No. 342,451, filed January 24, 1989 as PCT application No. PCT US88-03745, which is a continuation-in-part of Serial No. 113,014 filed October 23, 1987 and Serial No. 151,526, filed February 2, 1988.

TECHNICAL FIELD

This invention relates to the expression of <u>Vitreoscilla</u> hemoglobin in <u>Streptomyces sp</u>. to enhance growth characteristics and antibiotic yields at low or reduced oxygen levels.

This invention relates to the use of <u>Vitreoscilla</u> hemoglobin gene promoter to obtain high level expression of cloned proteins in <u>Streptomyces</u>.

BACKGROUND ART

The use of an intracellular globin to enhance growth and productivity in <u>Streptomyces</u> is based on several observations. First, the maximum cell concentration in <u>Streptomyces</u> fermentations is often limited by oxygen availability (Tuffile and Pinho, <u>Biotechnol. Bioeng.</u>, XII:849, 1970). Second, we have observed that in unicellular organisms there exists a possibly significant diffusional barrier between environmental

oxygen and the cytochromes where the energy-producing reactions necessary for cell growth occur. Third, the globins represent a family of heme-containing proteins that reversibly bind oxygen and are thus able to enhance the oxygen transfer rate to cells in multicellular organisms. Fourth, the synthesis of many antibiotics is reduced at low culture oxygen concentrations (Normansell, "Antibiotic-producing Streptomyces", The Bacteria, Queener and Day, Academic Press, N.Y., 1986). Finally, the expression of bacterial hemoglobin has been shown to enhance the growth properties of the bacteria Escherichia coli and Vitreoscilla, especially under conditions of reduced oxygen (Khosla and Bailey, Nature, 331:633, 1988). Expression of intracellular hemoglobin in Streptomyces may act to overcome the diffusional barrier, especially under conditions of low external oxygen, resulting in enhanced cell growth. Intracellular hemoglobin may also enhance antibiotic production per unit cell mass.

The bacteria of the genus Streptomyces are used for the production of approximately 60% of the commercially available antibiotics (Atkinson and Mavituna, Biochemical Engineering and Biotechnology Handbook, Macmillan, England, 1987). Examples of widely-used antibiotic compounds produced in Streptomyces fermentations include the spriamycins, neomycins, tetracyclines, and streptomycins (Demain and Solomon, Manual of Industrial Microbiology and Biotechnology, American Society for Microbiology, 1986). In addition, many compounds produced by Streptomyces have antineoplastic (the bleomycins, mithramycins, and daunomycins) and antihelminthic (the avermectins) activity. Recombinant DNA technology has been used to develop strains that overproduce or synthesize hybrid antibiotics with novel activities (Rhodes, et al., Biochem. Soc. Trans., 12: 1078, 1984). Through the use

of high-production strains and optimize growth protocols, the efficiency of antibiotic production can be dramatically improved. However, low antibiotic yields remain a major problem confronting industrial antibiotic production using Streptomyces.

Streptomyces are obligate aerobes that require high levels of oxygen for optimal growth. Providing sufficient oxygen to a high cell density culture represents a major obstacle due to the tendency for Streptomyces to form long filamentous strands that results in a highly viscous culture. Viscosity dramatically reduces the oxygen transfer rate to the culture medium. A typical Streptomyces fermentation begins with the growth of cells to high densities (growth phase). There is little antibiotic production during growth phase. The final cell densities achieved are usually limited by the oxygen supply. After cessation of growth, antibiotic synthesis begins (production phase). The antibiotic production phase is maintained as long as possible by supplying the appropriate nutrients. Eventually, acidic waste products accumulate and the cells die.

Although the factors regulating antibiotic production are largely unknown, certain environmental factors including phosphate and nitrogen concentration, carbon source, shear effects, and oxygen concentration have been shown to strongly influence antibiotic productivity. For example, in batch culture, cephalosporin production in S. clavuligerus dropped by a factor of three under reduced oxygen conditions (Yegneswaran, et al., Biotechnol. Letts., 10: 479, 1988). In addition, spectacular improvements in nikkomycin yields in S. tendae were achieved when the dissolved oxygen (DO) was maintained well above oxygen-limiting conditions during the production phase

(Aharonowitz and Demain, Biotechnolog: Potentials and Limitations, Springer and Verlag, Heidelberg, W. Germany, 1986). Unfortunately, maintaining sufficiently high DO levels in high density fermentations is technically difficult and often not economically feasible.

The mechanism by which reduced oxygen levels decreases antibiotic production in Streptomyces is unknown. One possibility is that lower respiration rates have a negative regulatory effect on secondary metabolic pathways (Vanek and Hostalek, Overproduction of Microbial Metabolites, Butterworth, MA, 1986). Aeration rates have also been shown to directly affect carbon source regulation of antibiotic synthesis (Brana, et al., Biotechnol. Letts., 5: 791, 1983). In addition, oxygen regulation of hydrolytic enzymes may play a role in antibiotic stability (Atkinson and Mavituna, ibid). Regardless of the mechanism, it is clear that it is desirable to facilitate oxygen transfer to the cells to increase antibiotic yields. Two general approaches to increasing the oxygen transfer rate to the culture medium include the development of improved bioreactor designs (Normansell, ibid.) and modification of the culture medium (Adlercreutz and Mattiason, Eur. J. Appl. Microbiol. Biotechnol., 16: 165, 1982).

The effect of bacterial hemoglobin expression on growth of a unicellular organism was investigated by Khosla and Bailey (Khosla and Bailey, ibid.). The bacterial hemoglobin was originally discovered in the obligate aerobic bacterium, Vitreoscilla (Tyree and Webster, J. Biol. Chem., 253: 6988, 1978). The hemoglobin is a soluble, dimeric protein that combines with oxygen and displays a spectral response to carbon monoxide binding characteristic of eukaryotic hemoglobins (Wakabayashi, et al., Nature, 332: 481, 1986). It was conjectured

that the hemoglobin protein functioned to facilitate oxygen transfer to Vitreoscilla and thus allowed it to propagate under oxygen-poor conditions.

The gene for the Vitreoscilla hemoglobin has been isolated along with its native transcriptional regulatory sequences. (Khosla and Bailey, Mol. Gen. Genet, 214: 158, 1988). Interestingly, this gene was expressed from its native promoter when introduced into E. coli. Of particular interest was that expression of hemoglobin was regulated by the culture oxygen content such that maximal induction occurred under microaerobic conditions. Under fed-batch fermentation conditions, E. coli cells expressing hemoglobin displayed significantly higher specific growth rates and achieved 2-3 fold the final cell densities as non-expressing strains (Khosla and Bailey, Nature, 331:633, 1988).

As mentioned previously, current progress in producing new antimicrobial compounds has involved the development, through recombinant DNA technology, of Streptomyces strains that produce novel "hybrid" antibiotics. So far, the expression of heterologous genes involved in secondary metabolite production has relied on the ability of the recipient strain to correctly recognize the transcriptional initiation sequence (promoter) of the incoming gene. The isolation of a universal, highly-active promoter for the expression of cloned genes in Streptomyces would be extremely useful, but has so far remained elusive.

DISCLOSURE OF THE INVENTION

The present invention relates to oxygen-binding proteins, particularly hemoglobins, a recombinant-DNA method of producing same, and to portable DNA sequences capable of directing intracellular production of these oxygen-binding proteins in Streptomyces. The present

invention also relates to vectors containing these portable DNA sequences.

One object of the present invention is to provide a recombinant-DNA method for the production of these oxygen-binding proteins. To facilitate the recombinant-DNA synthesis of these oxygen-binding proteins, it is a further object of the present invention to provide portable DNA sequences capable of directing intracellular production of oxygen-binding proteins in _Streptomyces_. It is also an object of the present invention to provide cloning vectors containing these portable sequences. These vectors are capable of being used in recombinant _Streptomyces_ to enhance the growth characteristics of organisms, and to produce useful quantities of oxygen-binding proteins. Augmented by intracellular synthesis of oxygen-binding proteins, product formation can also be enhanced.

The present invention also provides novel methods and materials for expression of cloned genes in _Streptomyces_. Particularly, it related to promoter/regulators, a recombinant-DNA method of producing same, and to portable DNA sequences capable of directing the translation and transcription initiation and control of the expression of desired gene products.

Thus, another object of the present invention is to provide for the expression in _Streptomyces_ of any selected chromosomal or extrachromosomal gene or DNA sequence through the incorporation of a promoter/regulator DNA sequence. Such expression may thus provide native or heterologous enzyme activities which increase antibiotic production or which enable synthesis of modified or novel antibiotics.

To achieve the objects and in accordance with the purposes of the present invention, promoter/ regulators are also set forth. To further achieve the objects in the accordance with the purposes of the present invention, as embodied and broadly described herein, portable DNA sequences for these promoter/regulators are provided. Particularly preferred promoter/regulator DNA sequences for use in the practice of the present invention are derived from the filamentous bacterium Vitreoscilla. Portable nucleotide sequences are provided for these promoter/regulators. The portable sequences may be either synthetic sequences or restriction fragments ("natural" DNA sequences).

Additionally, portable DNA sequences useful in the processes of the present invention may be synthetically created. These synthetic DNA sequences may be prepared by polynucleotide synthesis and sequencing techniques known to those of ordinary skill in the art.

Additionally, to achieve the objects and in accordance with the purposes of the present invention, a recombinant-DNA method is disclosed which results in manufacture by cells of the genus Streptomyces of the instant oxygen-binding proteins using the portable DNA sequences referred to above.

Additionally, to achieve the objects and in accordance with the purposes of the present invention, recombinant-DNA methods are disclosed which provide transcription and translation of gene products by a host Streptomyces using the portable DNA sequences referred to above.

To further accomplish the objects and in further accord with the purposes of the present invention, cloning vectors are provided comprising at least one portable

DNA sequence. In particular, plasmids pWLD5 and pWLD10 are disclosed.

It is understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

The accompanying drawing, which is incorporated in and constitutes a part of this specification, illustrates one embodiment of the invention and, together with the description, serves to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a partial restriction map of plasmids pWLD 10 and pWLD 5.

BEST MODES FOR CARRYING OUT THE INVENTION

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the drawing and the following examples, serve to explain the principles of the invention.

Expression of hemoglobin in Streptomyces serves to enhance aerobic growth, respiration, and/or antibiotic productivity. Thus, one objective of this invention is metabolically improved Streptomyces cells which have preferred functional characteristics in aerobic manufacturing processes. As noted above, the present invention relates in part to portable DNA sequences capable of directing intracellular production of oxygen-binding proteins in a variety of Streptomyces species. "Portable DNA sequence" in this context is intended to refer either to a synthetically produced nucleotide sequence or to a restriction fragment of a naturally occurring DNA sequence. For purposes of this

specification, "oxygen-binding protein" is intended to mean a protein with a primary structure as defined by the codons present in the deoxyribonucleic acid sequence which directs intracellular production of the amino acid sequence, and which may or may not include post-translational modifications. It is contemplated that such post-translational modifications include, for example, association with a heme prosthetic group. It is further intended that the term "oxygen-binding protein" refers to either the form of the protein as would be excreted from a cell or as it may be present in the cell from which it was not excreted. Because of the sensitivity of antibiotic synthesis in _Streptomyces_ to oxygen supply, it is also envisioned that the intracellular presence of cloned hemoglobin may provide a modified form(s) of the antibiotic molecule(s) normally produced by the host strain of _Streptomyces_. In a preferred embodiment, the portable DNA sequences are capable of directing intracellular production of hemoglobin. In a particularly preferred embodiment, the portable DNA sequences are capable of directing intracellular production of a hemoglobin biologically equivalent to that previously isolated from the filamentous bacterium, _Vitreoscilla_. By "biologically equivalent", as used herein, it is meant that a protein, produced using a portable DNA sequence of the present invention, is capable of binding oxygen in the same fashion, but not necessarily to the same degree, as the homodimeric soluble heme protein (subunit MW 15,775) isolable from _Vitreoscilla_.

As noted above, the present invention also relates in part to portable DNA sequences which contain promoter/regulators which are capable of directing intracellular expression of endogenous or exogenous gene products, in a variety of host cells and host microorganisms. "Portable DNA sequence" and

"promoter/regulator" in this context are intended to refer either to a synthetically produced nucleotide sequence or to a restriction fragment of a naturally occurring DNA sequence.

The portable DNA sequences of the present invention may also include DNA sequences downstream from a promoter/regulator which code for at least one foreign protein. For purposes of this specificatIon, "foreign protein" is intended to mean a protein with a primary structure as defined by the codons present in the deoxyribonucleic acid sequence which directs intracellular production of the corresponding amino acid sequence, and which may or may not include post-translational modifications. It is further intended that the term "foreign protein" refers to either the form of the protein as it would be excreted from a cell or as it may be present in the cell from which it was not excreted.

In a particularly preferred embodiment, the promoter/regulator contains transcription and translation initiation and control sequences substantially equivalent to those for directing intracellular production of a hemoglobin protein biologically equivalent to that previously isolated from the filamentous bacterium, <u>Vitreoscilla</u>.

It is of course intended that the promoter/ regulators of the present invention may control and initiate transcription and translation of an unlimited number of endogenous and/or exogenous foreign proteins. In particular, by expressing enzymes involved in antibiotic synthesis or modification, antibiotic productivity may be improved and the nature of the antibiotic may be modified.

A preferred portable DNA sequence for the promoter/regulators of the present invention contains at least a portion of the following nucleotide sequence, which reads 5' to 3' and includes the translation initiation sequence ATG (underlined) and some of the nucleotide sequence of the Vitreoscilla structural gene (also underlined):

Hin:

AAGCTTAACG GACCCTGGGG TTAAAAGTAT TTGAGTTTTG ATGTGGATTA AGTTTTAAGA 60

GGCAATAAAG ATTATAATAA GTGCTGCTAC ACCATACTGA TGTATGGCAA AACCATAATA 120

ATGAACTTAA GGAAGACCCT CATGTTAGAC CAGCAAACCA TTAACATCAT CAAAGCCACT 180

GTTCCTGTAT TGAAGGAGCA TGGCGTTACC ATTACCACGA CTTTTTATAA AAACTTGTTT 240

GCCAAACACC CTGAAGTACG TCCTTTGTTT GATATGGGTC GCCAAGAATC TTTGGAGCAG 300

CCTAAGGCTT TGGCGATGAC GGTATTGGCG GCAGCGCAAA ACATTGAAAA TTTGCCAGCT 360

ATTTTGCCTG CGGTCAAAAA AATTGCAGTC AAACATTGTC AAGCAGGCGR GGCAGCAGCG 420

CATTATCCGA TTGTCGGTCA AGAATTGTTG GGTGCGATTA AAGAAGTATT GGGCGATGCC 480

GCAACCGATG ACATTTTGGA CGCGTGGGGC AAGGCTTATG GCGTGATTGC AGATGTGTTT 540

ATTCAAGTGG AAGCAGATTT GTACGCTCAA GCGGTTGAAT AAAGTTTCAG
GCCGCTTTCA 600

GGACATAAAA AACGCACCAT AAGGTGGTCT TTTTACGTCT GATATTTACA
CAGCAGCAGT 660

TTGGCTGTTG GCCAAAACTT GGGACAAATA TTGCCCTGTG TAAGAGCCCG
CCGTTGCTGC 720
GACGTCTTCA GGTGTGCCTT GGCAT 745

The nucleotide bases represented by the above abbreviations are as follows: A = Adenine, G = Guanine, C = Cytosine, and T = Thymine.

The above sequence exhibits homology with certain sequences which are highly conserved in a variety of promoter/regulators. Using conventional numbering, with the underlining showing the homology in the above sequence to the consensus sequence, the -10 consensus sequence or Pribnow box sequence is TATAAT(A/G). The -35 consensus sequence is TTGACA, and the consensus Shine-Dalgarno sequence is AGGAGGTXXX(XX)ATG.

In a preferred embodiment, the above sequence is operatively fused with at least a portion of a downstream sequence of nucleotides which code for at least a portion of the Vitreoscilla hemoglobin protein which contains at least a portion of the following amino acid sequence:

5 10
Met-Leu-Asp-Gln-Gln-Thr-Ile-Asn-Ile-Ile-

15 20
Lys-Ala-Thr-Val-Pro-Val-Leu-Lys-Glu-His-

25 30
Gly-Val-Thr-Ile-Thr-Thr-Thr-Phe-Tyr-Lys-

35 40
Asn-Leu-Phe-Ala-Lys-His-Pro-Glu-Val-Arg-

45 50
Pro-Leu-Phe-Asp-Met-Gly-Arg-Gln-Glu-Ser-

55 60
Leu-Glu-Gln-Pro-Lys-Ala-Leu-Ala-Met-Thr-

65 70
Val-Leu-Ala-Ala-Ala-Gln-Asn-Ile-Glu-Asn-

75 80
Leu-Pro-Ala-Ile-Leu-Pro-Ala-Val-Lys-Lys-

85 90
Ile-Ala-Val-Lys-His-Cys-Gln-Ala-Gly-Val-

95 100
Ala-Ala-Ala-His-Tyr-Pro-Ile-Val-Gly-Gln-

105 110
Glu-Leu-Leu-Gly-Ala-Ile-Lys-Glu-Val-Leu-

115 120
Gly-Asp-Ala-Ala-Thr-Asp-Asp-Ile-Leu-Asp-

125 130
Ala-Trp-Gly-Lys-Ala-Tyr-Gly-Val-Ile-Ala-

135 140
Asp-Val-Phe-Ile-Gln-Val-Glu-Ala-Asp-Leu-

145 150
Tyr-Ala-Gln-Ala-Val-Glu

This amino acid sequence is disclosed in Wakabayashi _et al._, _supra_, _Nature_ 322:483, 1986. It is presently believed that the protein purified and prepared through the practice of this invention will exhibit a homology of over 80% with this sequence. The protein of this invention has been observed to enhance functioning of a cell in low oxygen environments (Khosla and Bailey, unpublished results).

The amino acids represented by the foregoing abbreviations are as follows:

Amino Acid	3-Letter Symbol
Glycine	Gly
Alanine	Ala
Valine	Val
Leucine	Leu
Isoleucine	Ile
Arginine	Arg
Lysine	Lys
Glutamic acid	Glu
Aspartic acid	Asp
Glutamine	Gln
Asparagine	Asn
Threonine	Thr
Serine	Ser
Cysteine	Cys
Methionine	Met
Phenylalanine	Phe
Tyrosine	Tyr
Tryptophan	Trp
Proline	Pro
Histidine	His

It must be borne in mind in the practice of the present invention that the alteration of some amino acids in a protein sequence may not affect the fundamental properties of the protein. Therefore, it is also contemplated that other portable DNA sequences, both those capable of directing intracellular production of identical amino acid sequences and those capable of directing intracellular production of analogous amino acid sequences which also possess oxygen-binding

activity, are included within the ambit of the present invention.

It must also be borne in mind in the practice of the present invention that the alteration of some nucleotide bases in a DNA sequence may not affect the fundamental properties of the coding sequence. Therefore, it is also contemplated that other analogous portable DNA promoter/regulator sequences are included within the ambit of the present invention.

It is contemplated that some of these analogous amino acid sequences will be substantially homologous to native Vitreoscilla hemoglobin while other amino acid sequences, capable of functioning as oxygen-binding proteins, will not exhibit substantial homology to native Vitreoscilla hemoglobin. By "substantial homology" as used herein, is meant a degree of homology to native Vitreoscilla hemoglobin in excess of 50%, preferably in excess of 80%.

Similarly, it is contemplated that some of these analogous DNA sequences will be substantially homologous to the sequence set forth above, while other DNA sequences, capable of functioning as the promoter/regulator described above, will not exhibit substantial homology to the sequence outlined above.

As noted above, the portable DNA sequences of the present invention may be synthetically created, by hand or with automated apparatus. It is believed that the means for synthetic creation of these polynucleotide sequences are generally known to one of ordinary skill in the art, particularly in light of the teachings contained herein. As examples of the current state of the art relating to polynucleotide synthesis, one is directed to Maniatis et al., Molecular Cloning--A

Laboratory Manual, Cold Spring Harbor Laboratory (1984), and Horvath et al., An Automated DNA Synthesizer Employing Deoxynucleoside 3'-Phosphoramidites, Methods in Enzymology 154:313-326, 1987, hereby incorporated by reference.

Additionally, the portable DNA sequence may be a fragment of a natural sequence, i.e., a fragment of a polynucleotide which occurred in nature. In one embodiment, the portable DNA sequence is a restriction fragment isolated from a genomic library. In this preferred embodiment, the genomic library is created from the bacterium Vitreoscilla. In other alternative embodiments, the portable DNA sequence is isolated from other genomic and cDNA libraries.

While it is envisioned that the portable DNA sequences of this invention may desirably be inserted directly into the host chromosome, the present invention also provides a series of vectors, each containing at least one of the portable DNA sequences described herein. It is contemplated that additional copies of the portable DNA sequence may be included in a single vector to increase a host cell's ability to produce large quantities of the desired oxygen-binding protein. It is also envisioned that other desirable DNA sequences may also be included in the vectors of this invention. Further, the invention may be practiced through the use of multiple vectors, with additional copies of at least one of the portable DNA sequences of this invention and perhaps other desirable DNA sequences.

In addition, the cloning vectors within the scope of the present invention may contain supplemental nucleotide sequences preceding or subsequent to the portable promoter/regulator and/or DNA sequence. These supplemental sequences are those that will not adversely

interfere with transcription of the portable promoter/regulator and/or any fused DNA sequence and will, in some instances, enhance transcription, translation, posttranslational processing, or the ability of the primary amino acid structure of the resultant gene product to assume an active form.

A preferred vector of the present invention is set forth in Figure 1. This vector, pWLD10, contains the preferred nucleotide sequence which codes for the amino acids set forth above. Plasmid pWLD10 (and pWLD5) may also contain supplemental nucleotide sequences such as terminators, enhancers, attenuators and the like. For proteins to be exported from the intracellular space, at least one leader sequence and any other DNA sequences necessary or preferred for appropriate transcription and subsequent translation of the vector DNA may be included within the scope of this invention.

In a preferred embodiment, cloning vectors containing and capable of expressing the portable DNA sequence of the present invention contain various operational elements in addition to or instead of the promoter/regulator disclosed and claimed herein. These "operational elements" may include at least one promoter, at least one sequence that acts as expression regulator, and at least one terminator codon, at least one leader sequence, and any other DNA sequences necessary or preferred for appropriate transcription and subsequent translation of the vector DNA.

Additional embodiments of the present invention are envisioned as employing other known or currently undiscovered vectors which would contain one or more of the portable DNA sequences described herein. In particular, it is preferred that these vectors have some or all of the following characteristics: (1) possess a

minimal number of host-organism sequences; (2) be stable in the desired host; (3) be capable of being present in a high copy number in the desired host; (4) possess a regulatable promoter; and (5) have at least one DNA sequence coding for a selectable trait present on a portion of the plasmid separate from that where the portable DNA sequence will be inserted. Alteration of vectors to meet the above criteria are easily performed by those of ordinary skill in the art in light of the available literature and the teachings herein. It is to be understood that additional cloning vectors may now exist or will be discovered which have the above-identified properties and are therefore suitable for use in the present invention and these vectors are also contemplated as being within the scope of this invention.

Any strain of Streptomyces which admits stable insertion of cloned DNA can serve as a host for the practice of this invention. Examples of Streptomyces strains which can be transformed or transduced are:

Streptomyces lividans 66 - Hopwood, et al., Genetic Manipulation of Streptomyces: A Laboratory Manual, The John Innes Foundation, Norwich, 1985.

Streptomyces coelicolor - Hopwood, et al, loc. cit.

Streptomyces parvulus - Hopwood, et al., loc. cit.

Streptomyces fradiae - Chung, J. Bacteriol., 169: 4436, 1987.

Streptomyces ambofaciens - Matsushima and Baltz, J. Bacteriol., 169: 4834, 1987.

Streptomyces griseofuscus - Larson and Hershberger, J. Bacteriol., 157: 314, 1984.

Streptomyces avermitilis - MacNeil and Klapko, J. Industr. Microbiol., 2:209, 1987.

Various vector systems will be suitable for Streptomyces species, including plasmids, and bacteriophages. The following, noninclusive list of cloning vectors is believed to set forth vectors which can easily be altered to meet the above criteria and are therefore preferred for use in the present invention. Such alterations are easily performed by those of ordinary skill in the art in light of the available literature and the teaching herein.

For example, the following Streptomyces plasmids have been used as vectors:

pIJ699 - Kieser and Melton, Gene, 65:83, 1988,

pIJ702 - Katz, et al., J. Gen. Microbiol., 129: 2703, 1983),

pHJL400 - Larson and Hershberger, Plasmid, 15: 199, 1986),

pKC505 - Richardson, et al., Gene, 61:231, 1987,

pSLP124 - Bibb and Cohen, Mol. Gen. Genet., 187: 265, 1982,

pSKO2 - Brawner, et al., Gene, 40:191, 1985,

pJAS14 - Forsman and Jaurin, Mol. Gen. Genet., 210:23, 1987, and

pARCI - Horinouchi and Beppu, J. Bacteriol., 162:406, 1985.

Phages used as Streptomyces vectors include derivatives of ϕC31 (Hopwood, et. al., Methods Enzymol., 153:116, 1987). See, for example, phage KC515 - Rodicio, et al., Gene, 34:283, 1985.

Synthesis and/or isolation of necessary and desired component parts of cloning vectors, and their assembly is believed to be within the duties and tasks performed by those with ordinary skill in the art and, as such, are capable of being performed without undue experimentation.

In construction of the cloning vectors of the present invention, it should additionally be noted that multiple copies of the promoter/regulator with any fused gene sequences and/or of the portable DNA sequence coding for the oxygen-binding protein and its attendant operational elements as necessary may be inserted into each vector. In such an embodiment, the host organism would produce greater amounts per vector of the cloned protein. The number of multiple copies of the DNA sequence which may be inserted into the vector is limited only by the ability of the resultant vector, due to its size, to be transferred into and replicated and expressed in an appropriate host.

Additionally, it is preferred that the cloning vector contain a selectable marker, such as a drug resistance marker or other marker which causes expression of a selectable trait by the host. In a particularly preferred embodiment of the present invention, the gene for thiostrepton resistance is included in vector pWLD10. Such a drug resistance or other selectable marker is intended in part to facilitate in the selection of transformants. Additionally, the presence of such a selectable marker on the cloning vector may be of use in keeping contaminating microorganisms from multiplying in the culture medium. In this embodiment, such a pure culture of the transformed host organisms would be obtained by culturing the organisms under conditions which require the induced phenotype for survival.

It is noted that the portable DNA sequence of the present invention may themselves be used as a selectable marker, in that they provide enhanced growth characteristics in low oxygen circumstances.

The promoter/regulators of this invention are capable of controlling expression of proteins or, thereby, of controlling synthesis of metabolites normally made by a cell, or of natural or unnatural metabolites and proteins expressed in a cell via genetic manipulation. This would include heterologous proteins--either intracellular or extracellular--as well as antibiotics and other chemicals produced by Streptomyces cells.

This invention also relates to a recombinant-DNA method for the production of oxygen-binding proteins. Generally, this method includes:

(a) preparing a portable DNA sequence capable of directing a Streptomyces host cell to produce a protein having oxygen-binding activity;

(b) transferring the portable DNA sequence directly into the host, or cloning the portable DNA sequence into a vector capable of being transferred into and replicating in the host cell, such vector containing operational elements for the portable DNA sequence;

(c) transferring the vector containing the portable DNA sequence and operational elements into the host cell capable of expressing the oxygen-binding protein;

(d) culturing the host cell under conditions appropriate for replication and propagation of the vector and/or expression of the protein; and

(e) in either order:
(i) harvesting protein, if desired; and
(ii) permitting the protein to assume an active structure whereby it possesses oxygen-binding activity.

It is envisioned that the portable DNA sequences may be inserted directly into the host chromosome, or alternatively may utilize a vector cloning system. The vectors contemplated as being useful in the present method are those described above. In a preferred embodiment, the cloning vectors pWLD10 and pWLD5 are used in the disclosed method.

A vector thus obtained may then be transferred into the appropriate <u>Streptomyces</u> species. It is believed that any <u>Streptomyces</u> species having the ability to take up exogenous DNA and express those genes and attendant operational elements may be chosen. Particular hosts which may be preferable for use in this invention include those described above. Methods for transfer of vectors into hosts are within the ordinary skill in the art. For ultimate expression in <u>Streptomyces</u>, it may be desirable that the cloning vector be first transferred into another microorganism such as <u>Escherichia coli</u>, where the vector would be allowed to replicate and, from which the vector would be obtained and purified after amplification, and then transferred into the <u>Streptomyces</u> for ultimate expression of the oxygen-binding protein.

The host cells are cultured under conditions appropriate for the expression of the oxygen-binding protein. These conditions are generally specific for the host organism, and are readily determined by one of ordinary skill in the art.

It is understood that application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use and manufacture appear below.

INDUSTRIAL APPLICABILITY

The products and processes of the present invention find usefulness in the production of antibiotics and the expression of any cloned proteins using Streptomyces in laboratory and industrial applications. The invention provides metabolically engineered cells with enhanced growth characteristics for increasing production of proteins, antibiotics, or other metabolites in Streptomyces. The invention also provides a DNA sequence that acts as a strong transcriptional initiation sequence for the expression of cloned proteins in Streptomyces.

EXAMPLE 1

Expression of a Bacterial hemoglobin in Streptomyces Enhances Cell Growth and Oxygen Uptake Rates under Oxygen-Limited Conditions.

A plasmid was constructed for the expression of a bacterial hemoglobin in Streptomyces. This plasmid, pWLD5, contains the Vitreoscilla hemoglobin gene and its native transcriptional regulatory sequences [Khosla and Bailey (1988) Mol. Gen. Genet., 214:158] cloned into a common Streptomyces plasmid, p1J699 [Keiser and Melton (1988) Gene, 65:83]. Specifically the 1.2 kilobase Hind III/SphI Vitreoscilla DNA fragment containing the hemoglobin gene was first inserted into the HindIII/SphI site of the Escherichia coli plasmid pUC19. This construct was then linearized with Hindiii and ligated

into HindIII-cut pIJ699. The resulting plasmid, pWLD5, was stably maintained in both E. coli and Streptomyces lividans.

S. lividans strain TK64 (pro2, str6, obtained from Dr. David Hopwood, John Innes Institute, Norwich, England) was transformed with pWLD5 DNA. A single thiostrepton-resistant colony, designated TK64:pWLD5, was selected for further experiments. Hemoglobin expression in TK464:pWLD5 was confirmed by Western analysis of total cell protein. A crude cell extract was generated by sonication and the proteins separated by SDS-polyacrylamide gel electrophoresis. The proteins were then electrotransferred to nitrocellulose membrane and screened with polyclonal antiserum generated against pure Vitreoscilla hemoglobin. A hemoglobin band of identical molecular weight as pure hemoglobin was detected in the cell extracts. Hemoglobin expression appeared to be constitutive as the levels were similar in cells sampled from any stage of growth. Expression of functional hemoglobin was demonstrated by a carbon monoxide difference spectrum technique [Webster and Liu (1974) J. Biol. Chem. 249:4257].

To investigate the effect of hemoglobin expression on cell growth and respiration, TK64:pWLD5 was compared with the plasmid-free strain (TK64) under two culture conditions corresponding to high and low aeration. The culture medium used for the experiment was as follows: 3% dextrose, 2% N-Z amine Type I, 1% yeast extract, and 1% v/v trace elements mix (0.1% $FeSO_4 \cdot 7H_2O$, 0.1% $MnSO_4 \cdot 7H_2O$, 0.0025% $CuCl_2 \cdot 2H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.00056% H_3BO_3, 0.002% $ZnSO_4 \cdot 7H_2O$, 0.0019% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$). 5 ug/mL of thiostrepton was added to the TK64:pWLD5 culture. The first condition (high aeration) was a 50 mL culture volume in a 250 mL unbaffled erlenmeyer flask shaken at 250 rpm at 300C. The second condition (low

aeration) was a 75 culture volume in a 250 mL unbaffled erlenmeyer flask shaken at 150 rpm at 30°C. With high aeration, the two strains had similar maximum specific growth rates (0.22-0.24 h^{-1}) but the plasmid-free strain reached a higher final cell density (OD_{590}=7.0) compared to TK64:pWLD5 (OD_{590}=5.0). With lower aeration, however, TK64:pWLD5 reached a higher final cell density (OD_{590}=1.95) than the plasmid-free strain (OD_{590}=1.25). This represents a 56% increase in the final cell density in cells expressing hemoglobin under reduced aeration conditions. The maximum specific growth rates of the two strains were similar (0.10-0.11 h_{-1}) under reduced aeration. Hemoglobin expression levels in the two strains were similar throughout the experiment as demonstrated by Western analysis.

Oxygen uptake rates (OUR's) were compared between TK64:pWLD5 and the plasmid-free strain throughout this experiment. Cells were removed at various times, washed, and resuspended in fresh medium at an OD_{590} of 0.10. The OUR's were then measured using a Yellow Springs instruments biological oxygen monitor. The rates were normalized to cell weights and compared throughout the growth curve (Table 1). Although the OUR's of the two strains were similar throughout the experiment with high aeration (Table 1A), they were consistently higher in the hemoglobin-expressing strain with lower aeration, especially at the later stages of growth (Table 1B). For example, at an OD_{590} of approximately 0.6, the OUR for the plasmid-free strain was 0.22 mM O_2/h-g whereas the OUR for TK64:pWLD5 was 0.29 mM O_2/h-g, a difference of 32%.

This experiment indicates that <u>Streptomyces</u> cells expressing a bacterial hemoglobin grow to significantly higher cell densities and have higher oxygen uptake rates than the non-expressing strain under reduced

aeration conditions. A similar plasmid, pWLD15, containing the same Vitreoscilla hemoglobin gene (including its transcriptional regulatory sequence) fragment as that in pWLD5, except that it was cloned into the opposite orientation, also expresses hemoglobin in Streptomyces. This latter finding is evidence that the expression of the hemoglobin gene originates in the inserted fragment (originating from Vitreoscilla) as opposed to elsewhere on the Streptomyces-based pIJ699 plasmid.

Table 1A - High aeration

Strain	$O.D._{590}$	O.U.R. (mM O_2/h-g)
TK64	0.5	0.32
TK64:pWLD5	0.4	0.35
TK64	0.9	0.32
TK64:pWLD5	0.8	0.33
TK64	5.0	0.11
TK64:pWLD5	4.6	0.12

Table 1B - Low aeration

Strain	$O.D._{590}$	O.U.R. (mM O_2/h-g)
TK64	0.3	0.35
TK64:pWLD5	0.3	0.42
TK64	0.6	0.22
TK64:pWLD5	0.6	0.29
TK64	2.0	<0.10
TK64:pWLD5	2.0	0.27

EXAMPLE 2

Growth enhancement of hemoglobin-expressing Streptomyces grown under two additional conditions of reduced oxygen.

The enhanced growth of hemoglobin-expressing Streptomyces was examined under two additional conditions of low aeration in shakeflask cultures. Strains TK64 (no plasmid) and TK65:pWLD5 were cultured

in 12.5 and 25 mL culture volumes in 250 mL flasks for 72 hours at 150 rpm at 30°C. The medium used was the same as in Example 16. The final cell densities were measured at OD_{590}. in the 12.5 mL culture, TK64:pWLD5 reached a final OD_{590} of 5.8 while TK64 reached an OD_{590} of only 4.0, a difference of 45%. In the 25 mL culture, TK64:pWLD5 reached a final OD_{590} of 4.5, while TK64 reached an OD_{590} of only 3.3, a difference of 41%. This experiment indicates that hemoglobin expression benefits Streptomyces cell growth under two additional conditions of reduced culture oxygen.

EXAMPLE 3

Expression of bacterial hemoglobin in Streptomyces coelicolor.

To demonstrate that Vitreoscilla hemoglobin can be expressed in another streptomycete, a plasmid similar to pWLD5 was constructed by inserting BamHI-linearized pRED2 [Khosla and Bailey (1988) Mol. Gen. Genet. 214:158] into BgIII-digested p1J699. pRED2 contains the identical hemoglobin sequence as pWLD5 but contains an additional 1.5 kb of non-essential DNA. The resultant plasmid, PWLD10, was transformed into Streptomyces coelicolor strain M145 (SCP1^{-}, SCP2- obtained from Dr. David Hopwood, John Innes Institute, Norwich, England) and a single thiostrepton-resistant transformant, designated M145:pWLD10, was selected for further experiments.

M145:pWLD10 cells were grown in liquid culture to exponential phase in 50 mL YEME medium (0.3% yeast extract, 0.5% peptone, 0.3% malt extract, 1% glucose, 34% sucrose, 5 mM $MgCl_2 \cdot 6H_2O$) at 250 rpm at 30°C. A cell extract was prepared by sonication and the proteins separated by SDS-PAGE and screened with anti-Vitreoscilla hemoglobin antisera. Western analysis

indicated that a significant level of hemoglobin of identical molecular weight as pure Vitreoscilla hemoglobin was present in cell extracts of M145:pWLD10 but not in the plasmid-free strain. This indicates that Vitreoscilla hemoglobin is stably expressed in another species of Streptomyces.

These data also indicate that the Vitreoscilla hemoglobin promoter element functions in S. coelicolor to express a heterologous protein. Thus, this promoter functions in different strains of Streptomyces.

EXAMPLE 4

Expression of Bacterial Hemoglobin in Streptomyces coelicolor Results in Higher Final Antibiotic Levels.

Antibiotic production in Streptomyces coelicolor strains M145 and M145:pWLD10 was compared in a shake flask culture experiment. One mL of exponential phase cells were inoculated into 50 mL of YEME medium (5 ug/ml thiostrepton was added to the M145:pWLD10 culture) in 250 mL unbaffled flasks. The cells were grown at 250 rpm at 30°C. Ten days later the cultures were analysed for the production of the pigmented antibiotic, undecylprodigiosin. The assay was performed by mixing equal volumes of the culture and 0.1 M NaOH followed by a 30" sonication (50 Watt output) on ice. The sonicate was then filtered through a 0.2 uM nitrocellulose membrane. The OD_{468} of the filtrate, which is a measure of undecylprodigiosin, was then determined. While the hemoglobin-expressing strain had an OD_{468} of 1.4, the non-expressing strain had an OD_{468} of only 0.6. This indicated that greater than twice as much antibiotic is produced in a hemoglobin-expressing strain of Streptomyces.

WHAT IS CLAIMED IS:

1. A recombinant-DNA vector capable of directing intracellular production in Streptomyces of at least a portion of the Vitreoscilla hemoglobin.

2. A vector according to Claim 1 capable of said production of Vitreoscilla hemoglobin in Streptomyces lividans.

3. A recombinant-DNA vector comprising the Vitreoscilla hemoglobin promoter as an expression initiation signal capable of directing intracellular production in Streptomyces of a heterologous protein.

4. A vector according to Claim 3 wherein said Streptomyces comprises Streptomyces lividans.

5. A vector according to Claim 1 or 3 wherein said Streptomyces coelicolor.

6. A recombinant-DNA method for production of at least a portion of the Vitreoscilla hemoglobin protein in a Streptomyces host grown in the presence of oxygen, comprising:

(a) introducing a vector capable of directing intracellular production in Streptomyces of at least a portion of Vitreoscilla hemoglobin protein into said Streptomyces host; and

(b) culturing said host under conditions appropriate for expression of said protein.

7. A method according to Claim 6 wherein said host comprises Streptomyces lividans.

8. A method according to Claim 6 wherein said host comprises Streptomyces coelicolor.

9. A protein prepared by the method of Claims 6 or 7 comprising at least a portion of the _Vitreoscilla_ hemoglobin protein.

10. Recombinantly modified _Streptomyces_ containing a vector according to Claim 1 and capable of intracellular production of at least a portion of the _Vitreoscilla_ hemoglobin.

11. Recombinantly modified _Streptomyces lividans_ according to Claim 10.

12. Recombinantly modified _Streptomyces coelicolor_ according to Claim 10.

13. A method for expressing in _Streptomyces_ a selected chromosomal or extrachromosomal gene or DNA sequence comprising the steps of

(a) introducing into a _Streptomyces_ host cell capable of expressing said selected gene or said sequence, a vector capable of directing intracellular production in _Streptomyces_ of at least a portion of _Vitreoscilla_ hemoglobin protein into said host cell;

(b) introducing, if necessary, into said host cell a second vector capable of directing intracellular expression of said selected gene or said selected DNA sequence in said host cell; and

(c) culturing said host under conditions appropriate for expression of said selected gene or DNA sequence and for production of said protein.

14. A method according to Claim 13 wherein said host comprises _Streptomyces lividans_.

15. A method according to Claim 13 wherein said host comprises _Streptomyces coelicolor_.

16. A method according to Claim 15 wherein said selected gene is a chromosomal gene and said expression produces an antibiotic.

17. A method according to Claim 16 wherein said antibiotic comprises undecylprodigiosin.

18. A method for expressing in Streptomyces a selected chromosomal or extrachromosomal gene of DNA sequence comprising the steps of:

(a) introducing into a Streptomyces host cell capable of expressing said selected gene or sequence, a vector comprising the Vitreoscilla hemoglobin promoter as an expression initiation signal for expression of said gene or sequence;

(b) culturing said host under conditions appropriate for expression of said selected gene or sequence.

19. A method according to Claim 18 wherein said host comprises Streptomyces lividans.

20. A method according to Claim 18 wherein said host comprises Streptomyces coelicolor.

21. A method for increasing cell density of a Streptomyces host culture comprising the steps of:

(a) introducing into said host a vector capable of directing intracellular production in Streptomyces of at least a portion of Vitreoscilla hemoglobin protein;

(b) culturing said host under conditions appropriate for expression of said protein.

22. A method according to Claim 21 wherein said Streptomyces comprises Streptomyces lividans.

23. A method according to Claim 21 wherein said <u>Streptomyces</u> comprises <u>Streptomyces coelicolor.</u>

24. A method for increasing the respiration rate during growth of a <u>Streptomyces</u> host culture comprising the steps of:

(a) introducing into said host a vector capable of directing intracellular production in <u>Streptomyces</u> of at least a portion of <u>Vitreoscilla</u> hemoglobin protein;

(b) culturing said host under conditions appropriate for expression of said protein.

25. A method according to Claim 24 wherein said <u>Streptomyces</u> comprises <u>Streptomyces lividans.</u>

26. A method according to Claim 24 wherein said <u>Streptomyces</u> comprises <u>Streptomyces coelicolor.</u>

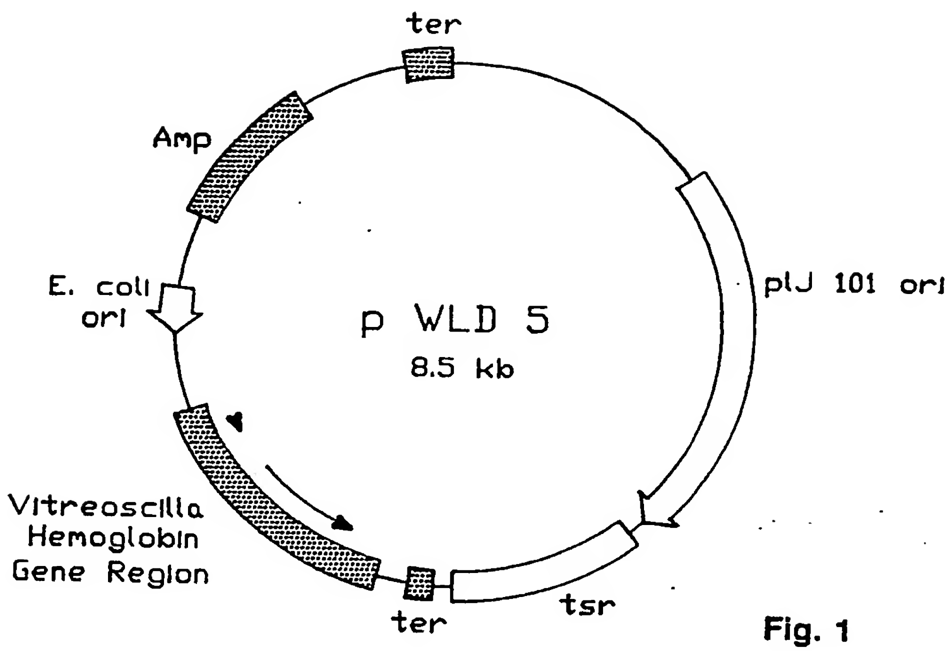
27. A method for increasing antibiotic productivity of an antibiotic-producing <u>Streptomyces</u> host culture comprising the steps of:

(a) introducing into said host a vector capable of directing intracellular production in <u>Streptomyces</u> of at least a portion of <u>Vitreoscilla</u> hemoglobin protein;

(b) culturing said host under conditions appropriate for expression of said protein.

28. A method according to Claim 27 wherein said <u>Streptomyces</u> comprises <u>Streptomyces lividans.</u>

29. A method according to Claim 27 wherein said <u>Streptomyces</u> comprises <u>Streptomyces coelicolor.</u>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Amp
E. coli ori
p WLD 5
8.5 kb
pIJ 101 ori
Vitreoscilla Hemoglobin Gene Region
ter
tsr

Fig. 1

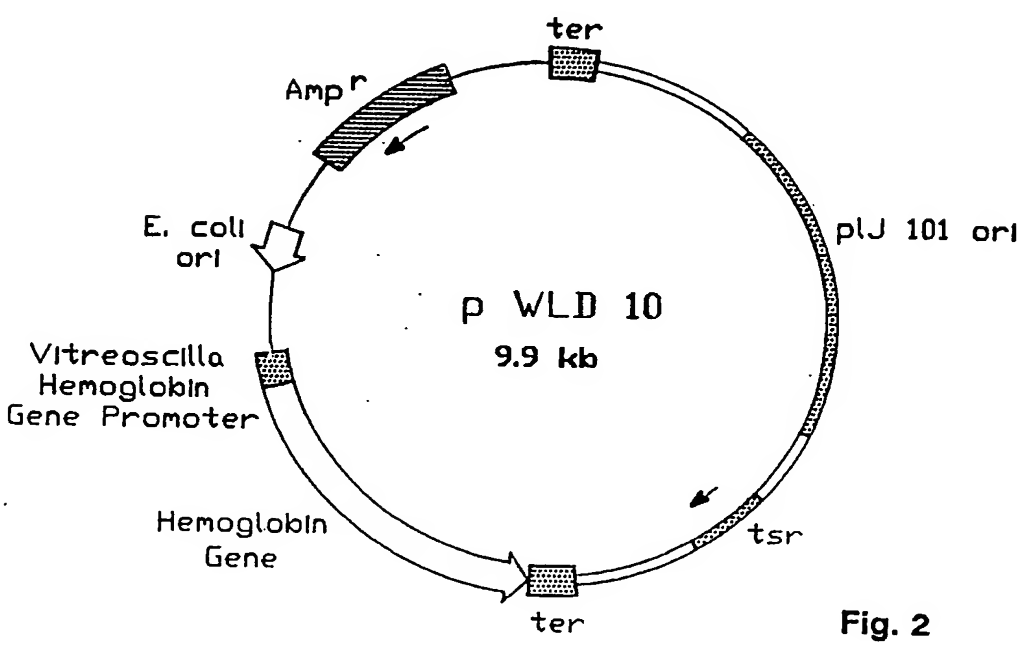
ter
Amp r
E. coli ori
p WLD 10
9.9 kb
pIJ 101 ori
Vitreoscilla Hemoglobin Gene Promoter
Hemoglobin Gene
tsr
ter

Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/06081

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC(5): C12N 1/21, 15/21, 15/09, 15/76, 15/67
U.S. CL.: 435/252.35; 536/27; 435/69.1; 320.1 172.3

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S,.	435/69.1, 71.1, 72, 91, 172.3, 252.35, 320.1, 886; 536/27; 935/6, 9, 22, 29, 38, 59, 60, 61, 66, 75

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical Abstracts Database (CA):1967-1991; Biosis Database 1969-1991
Keywords: Streptomyces; Vitreoscilla; oxygen (uptake, tension); hemoglobin; antibiotic; fermentation

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	MOLECULAR AND CELLULAR GENETICS, Volume 214, No. 1, issued September 1988, C. KHOSLA ET AL., "The Vitreoscilla hemoglobin gene: Molecular cloning, nucleotide sequence and genetic expression in Escherichia coli", pages 158-161, see entire document.	1-29
Y	NATURE, Volume 331, issued 18 February 1988, C. KHOSLA ET AL. "Heterologous expression of a bacterial haemoglobin improves the growth properties of recombinant Escherichia coli", pages 633-635, see entire document.	1-29
Y	BIOLOGICAL ABSTRACTS, Volume 87, No. 2, issued 15 January 1989, Rollins et al., "Effect of aeration on antibiotic production of Streptomyces clavuligerus see abstract no. 15512, J. Ind. Microbiol. 3(6): 357-364.	1-29

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12 February 1991	11 MAR 1991

International Searching Authority	Signature of Authorized Officer
ISA/US	Richard C. Peet Richard C. Peet

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	GENE, Volume 65, issued June 1988, T. KIESER ET AL., "Plasmid pIJ699, a multi-copy positive-selection vector for Streptomyces", pages 83-91. see entire document.	1-29